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COMPARATIVE ANALYSIS OF PULSED (ACTIVATED) AND RESTING CHICKEN CYTOCHROME C OXIDASES WITH SODIUM CHLORIDE DEPENDENCE OF THE ENZYME

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ABSTRACT

Comparative investigation of pulsed (activated) chicken cytochrom c oxidase and resting enzyme are compared with other species and pH dependent properties are also monitored Sodium chloride dependence of chicken oxidase is analysed and compared with that of beef cytochrome c oxidase. Carbon monoxides binding and spectral changes during this process is also discussed.

S values are also determined at different pH of the enzyme ultra centrifugation.

INTRODUCTION

Cytochrome c oxidase (Ec 1.9.3.1) is one of the most important enzyme in nature being involved in terminal oxidative steps in energy metabolism. It is widely investigated enzyme. Cytochrome c oxidase plays central role in the aerobic metabolism of the obligative aerobes. Cytochrome c oxidase is a metalloenzyme containing two haems a and a_3 and two copper atoms. The enzyme is ambeded in inner mitochondria) membrane (Azzi A., 1980) and catalyses the passage of electron from single site redox protein cytochrome g to oxygen in multiple electron steps, the mechanism of the enzyme is subject of considerable research effort (Lemberg A., 1969). It has been observed that various procedures applied to prepare this enzyme varies in ratio of purity (i.e.) proteins, lipids, haemes, iron. This enzyme has seven to nine sub units on SDS gel with different molecular weights. Its physical and chemical properties are identical (Alizai M.N., 1990, 1995). We have isolated chicken cytochrome c oxidase from chicken hearts and compared it with beef and other species (Nicholis P., 1974, Okunuki K., 1972). Cytochrome c oxidase can be activated by introducing oxygen into fresh enzyme and activity can be increased five to ten folds. An activated or oxygenated cytochrome oxidase as described by O, Kumuki (Okunuki K., 1968) had received a great deal of attention. Many workers believed that it was in fact an intermediate involved in catalytic mechanism of cytochrome c oxidase. Evidence has been amassed to show that resting enzyme or oxidized-oxidase as prepared is not the form which takes part in enzyme turnover. After some cyclation of oxidation and reduction an activated enzyme is produced. This has been characterized and named as pulsed oxidase by Antonini and Brunori (Antonini E., 1997, Brunori M., 1979). They investigated the significance of pulsed oxidase and defined them as the

species which are obtained by the exposure of fully reduced enzyme to pulse of oxygen. We have conducted the stopped flow kinetic experiments for resting, oxygenated, pulsed chicken and beef cytochrome c oxidases for comparative purpose. We have also comparatively investigated NaCl effect on chicken and beef oxidase with pH dependence of the oxidases. The molecular weight of the enzymes were determined by gel chromatography (Alizai M.N., 1997).

MATERIAL AND METHODS

Preparation of cytochrome c oxidase

Chicken and beef cytochrome c oxidase were prepared using hearts tissue following Yonetani and Capaldi (Yonetani T., 1960; Capaldi R.A., 1982) methods. The method of Yonetani (1960) was further modified and established as Alizai method in these Laboratories. The principle steps in preparation were (i) preparation of Keilin-Hartree particles and solubilization in 2% cholate buffer (Yonetani T., 1960) (ii) removal of cytochrome b and c in successive ammonium sulphate fractionations. The progress of preparation was monitored by spectroscopy. The purified enzyme was dissolved in 0.1 M sodium phosphate buffer pH 7.4 containing 1% Tween 80 (Darley U.V.M., 1981).

Sample of oxidases were purified through cepharose 4B and 6B column separately (2 x 40cm) equilibrated with 0.1 M sodium phosphate buffer at pH 7.4 containing 1% Tween 80 at 4°C. The eluant was monitored at 280 nm. The purified oxidase fractions were collected after activity assays and kept at -20°C for further investigation (i.e.) pulsed oxidase, pH dependence, S.D.S. gel electrophoresis and later treated with enzymes inhibitors like trypsin, chymotrypsin, cholic acid etc. (Malmstrom B.G., 1979).

Ultracentrifugation:

Ultracentrifugation was conducted using Backman Model E analytical centrifuge (Lov V., 1970). Protein concentration was 10 mg/ml and samples were solubilized in 0.1 M sodium phosphate buffer, pH 7.4 containing 1% Tween 80. Computerized S values were calculated to find molecular weight of the oxidase.

Steady Stare Activity Measurements:

Oxidase activities were measured polarographically or spectrophotometrically using oxygen electrode (Rank brothers Cambridge England) or Parkin Elmer 575 spectrophotometer with scanner fitted with a scattering sample accessory and background corrector. Assays were conducted in either high ionic concentration of Sodium phosphate buffer (p = 0.2) at pH 7.0, containing 0.1% Tween 80 or low ionic strength.buffer 25mM Cacodylate/HCI pH 7.0, 250 mM sucrose 0.5% Tween 80 and 1 mM EDTA. Concentration of horse heart cytochrom c were varied from 0.01 to 30 μM, sodium ascorbic (3.3 mM) was used as the reducing agent and TMPD (NN'N'N'- Tetramethyl-p-phenylenediamine hydrochloride) 0.7 mM as a mediator. Oxidase concentrations varied between 0.1 μM -1 μM (Functional unit). The cytochrom c was fully reduced during course of the assay was checked spectrophotometrically. Activity as a function of pH was measured over a range of pH values using Sodium phosphate buffer of ionic strength (μ = 0.2). The final concentration of cytochrome c and oxidase was 30 mM and 1 μM (FM) respectively.

Kinetic Measurements:

Rapid mixing experiments were performed using Gibson-Durrum stopped flow apparatus (Technation Ltd. Edgeware Middlesex UK) fitted with 2 cm light path. This instrument was first scribed by Gibson and Milne (Gibson Q.H., 1964). Light was provided using either a deuterium or Tungsten Lamp depending upon the wave length to be studied. The wavelength required was selected by a Baush Lamb monochrometer and curve were displayed on oscilloscope screen. Dead time of the apparatus, that is the time between mixing the reactants and observing the reaction was approximately 2-3 milli seconds. The light emitted from observation chamber impinged upon a photomultiplier, the out put of which was recorded on the screen of oscilloscope. Permanent data of the traces were obtained by photographing the oscilloscope screen with polaroid land film type 107C. The photographs were subsequently traced on to the graph paper for analysis.

Spectral changes of Enzyme:

pH of the enzyme solution containing sodium phosphate-buffer pH 7.4 with 1% Tween 80 was raised by continuous addition of 0.1 N sodium hydroxide dropwise while stirring the oxidase at 4°C. At each pH oxidase was collected and spectra were taken using Perkin Elmer 575 spectrophotometer. Enzyme was solubilized in 0.1 M sodium phosphate buffer pH 7.4, 8.5, 9.0, 9.5, 10.0, 10.5 and pH 11.0. The spectra were analysed by observing at 440 nm or 605 nm.

S.D.S. gel electrophoresis were also performed for each of the collected sample separately.

RESULTS AND DISCUSSION

The stopped flow apparatus was used to study the oxidation of ferrocytochrome c by chicken oxidase under the following experimental condition.

Oxidase [4 μM]
Ferrocytochrome c [10 μM] "Resting" enzyme
Ascorbate [300 μM]

Oxygen [135 μM]

Ferrocytochromec [10 μM] "Resting" enzyme
Ascorbate [300 μM]

Ferrocytochrome c was prepared using the sephadex G25 column for purification after reducing with sodium dithionite.

By taking advantage of the fact that reaction of reduced cytochrome c oxidase with molecular oxygen is very fast (Green Wood 1975, Chance 1975), it is still possible to investigate the kinetic properties of this reaction immediately after formation of the reaction product (pulsed oxidase). In stopped flow experiments, pulsed cytochrome c oxidase was exposed to reduced cytochrome c in absence or in presence of excess O_2. The results are compared side by side with the results of similar experiments using cytochrome c oxidase as prepared termed as resting oxidase (See Fig.l). It can be seen from Fig. 1 that constant concentration of reduced cytochrom c and at high oxidase

concentration, the initial velocity for the steady state phase was much faster starting with pulsed enzyme than starting with resting oxidase. The difference being approximately 5 to 6 folds. These results were in agreement with those already reported initially by Antonini (Antonini E., 1971) and then Bmnori (Brunori M., 1979) Darleyusmar (Darleyusmar V.M., 1990) for bovine oxidase. Sodium chloride dependence of pseudo first order rate constant (K) for the slow phase of cytochrome c oxidation observed (see Fig. IB) $[NaCl]_{50}$ refer to the molar concentration of sodium chloride required to reduce to 50% of its initial value. The concentration of oxidase were 1.5 mM (F.U) and ferrocytochrome c concentration 50 M. Experiments were performed in Tris/HCI buffer, ionic strength p=0.08 at 25°C. NaCl was added to both cytochrome c and oxidase before mixing in the stopped flow apparatus. The experimental design ensured that while the [NaCl] was varied the protein concentration remained constant. The monitoring wave length was 550 nm.

We have conducted assays of chicken oxidase as prepared, treated with trypsin, chymotrypsin and after gel chromatography. Spectrophotometric method was used. Enzyme was solubilized in 0.1 M phosphate buffer pH 7.4 containing 1% Tween 80. Data is summarized in Fig 2. Oxygen electrode was also used for comparative purpose and freshly prepared chicken oxidase and purified through sepherose 4B were applied. The initial rate v is plotted in the formation of Eadie-Hoftsu V/S versus v/v where V/S is the initial ferrocytochrome c concentration (see Fig. 3). Activated are expressed as moles of cytochrom c per moles of oxidase (F.U) per min^{-1} (Ludwig B., 1979).

Absolute spectra of chicken oxidase were taken at different pH value of the enzyme from pH 7.0 to pH 11.0. Catalytic activity shows that oxidase at or above pH 9.5 become two to six fold more active than at the neutral pH of the enzyme. Monomers were separated from dimers at pH 10.0 which show the highest catalytic activity in terms of (TN) Turnover number as pM of cytochrom c μM of oxidase/Sec^{-1} (see Fig. 4).

Fig. 5A presented here indicated typical chicken oxidase spectra, oxidised, reduced and reduced carbon-monoxide binding. Oxidase was solublised in 0.1 M sodium phosphate buffer pH 7.4 containing 1% Tween 80. Other spectra were shown at pH 9.0, pH 9.5, pH 10.0, pH 10.5 and pH 11.00. Changes in spectra confirmed the fact that enzyme dissociate to monomer above pH 9.5 and part of the enzyme denature with addition of alkali. The changes can well be noticed in spectra 5E and 5F. Denatured protein can be separated from monomers through gel chromatography.

Ultracentrifugation data confirm that enzyme remains dimer at physiological pH while on alkalination dimers started dissociating into monomers. Photographs presented in Fig. 6 (ABCD) shows the pattern of sedimentation. S values were calculated by graph as Lnr (as the distance from the centre of the rotor to the sedimentation peak) VS time (Gutweniger H., 1981).

$$S = \frac{\text{Slope}}{W^2} \qquad \text{where } W^2 = \frac{rPmx27}{60}$$

where S is the sedimentation coefficient and W is the angular velocity of the centrifuge head (see Fig. 7) Results were calculated by computer (Table).

CONCLUSION

Comparative analysis of molecular weight (S values), the steady state and transient kinetic of the chicken enzyme including pulsed and resting enzyme, plobable sequence of the redox centre in electron transfer mechanism and monomers formation on alkalination of dimeric enzyme led to the conclusion that structurally, functionally, eukaryotic cytochrome oxidase are remarkably conservative.

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Table
Some of the Computerized results for pH dependent S value of chicken oxidase

PH	S Value	Slope
7.4	11.5	.267
9.0	7.64	.226
9.5	5.57	.165
10.0	5.38	.159
10.5	4.46	.132
10.8	3.66	.108
11.5	1.64	.048

Results were unconnected for lipid or solvent density
Rotor speed 52000 rpm (round per minute) correlation ~ 0.98 to 0.99.

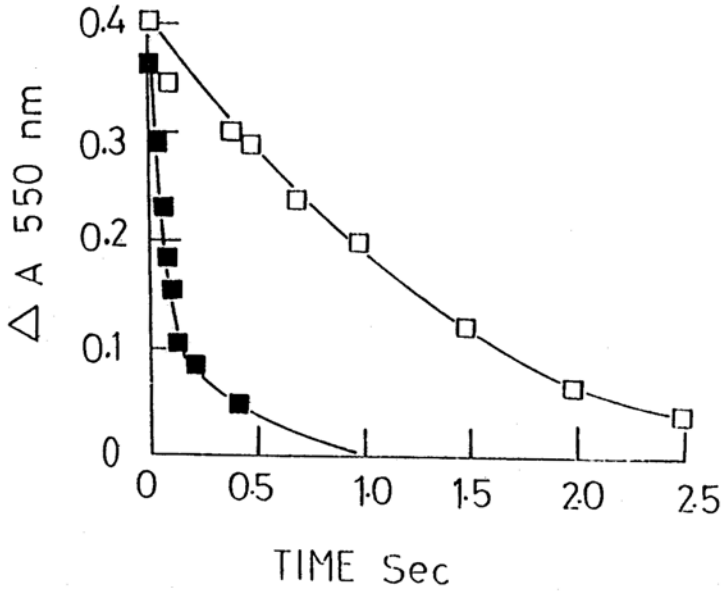

Figure l(A): Progress curves for slow phase of cytochrome c oxidation monitored at 550 nm. Cytochrome c concentration was 30 μM and oxidase concentration 3 μM (F.U) Reaction were performed in stopped flow apparatus. Oxidase was dissolved in 0.1 M sodium phosphate buffer pH 7.4 containing 1% Tween 80. □ indicate resting enzyme ■ indicate pulsed (activated) enzyme.

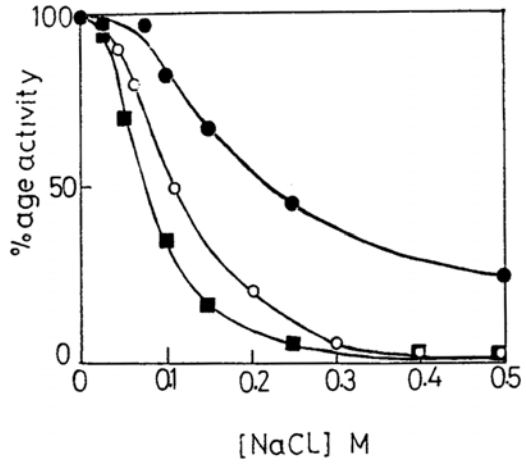

Figure 1(B): Salt concentration dependence of slow oxidase of ferrocytochrome c catalysed by chicken, dog fish and beef oxidases. The reaction was followed at 550 nm in stopped flow apparatus. Ferrocytochrome c concentration was 6 uM ♦ indicated 7.4 ♦ indicated chicken oxidase (2 μM concentration) in same buffer and Dog fish oxidase was indicated by ● (2 μM concentration in the buffer stated above. All buffer containing 1% Tween 80. Temperature was 20c.

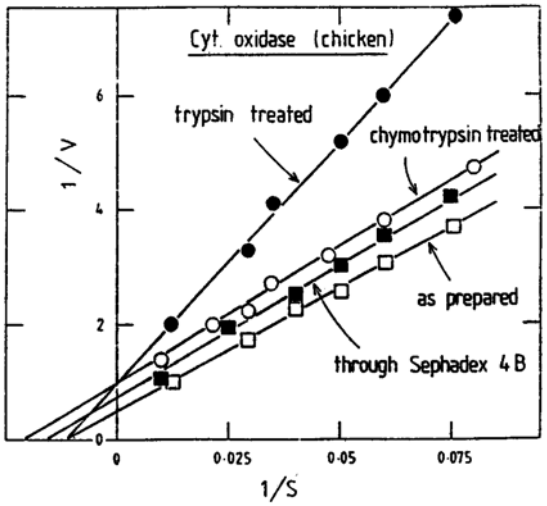

Figure 2: Activities of chicken cytochrome c oxidase treated with trypsin, Chymotrypsin, purified through sepharose 4B, and fresh as prepared. Activities were measured spectrophotometrically. Oxidase was assayed by following the oxidation of horse heart cytochrome c 550 nm using Shimadzu U.V. 190, spectrophotometer. Buffer used in assay was 0.1 M sodium phosphate pH 7.4 containing 1% Tween 80.Spectrophotometric results were plotted in form of linweaver Burk double reciprocal plots from which Vmax and turnover number (TN) were calculated. V is expressed in pM of cytochrom c.

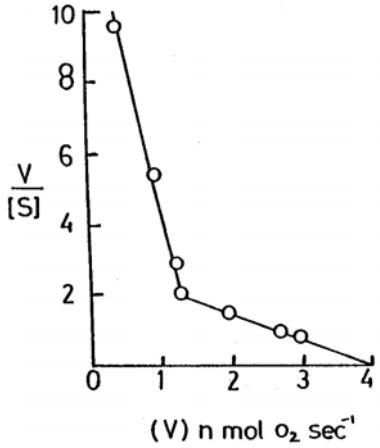

Figure 3: Eadie-Hoftsee plot for the rate of oxygen consumption of purified solubilized chicken cytochrome c oxidase using horse heart cytochrome c as substrate. Chicken oxidase was in 0.1 M sodium phosphate buffer pH 7.4 containing 1% Tween 80. The concentration of horse heart cytochrom c was varied from 0.04 - 30 μM, oxidase concentration was 0.2 μM (F.U.) final concentration.

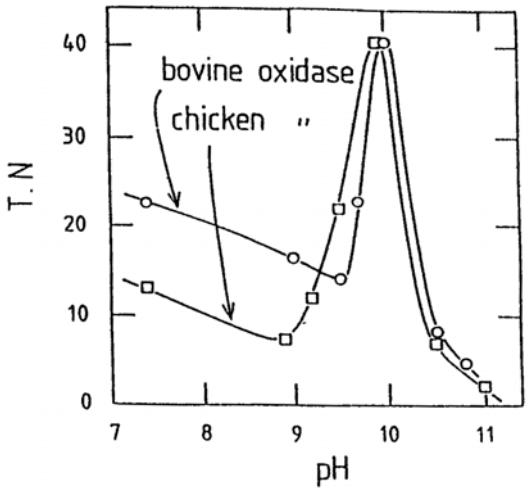

Figure 4: Activities of bovine and chicken oxidase was measured by spectrophotometric method at different pH. The pH of the enzyme was raised from neutral to pH 11.0. Assays were done in 0.1 M sodium phosphate buffer pH 7.4 containing 1% Tween 80. Linweaver-Burk double reciprocal plots were plotted and Turnover number (T.N) were obtained. Graphs of T.N VS pH of both enzyme were drawn as shown in Fig. 4. T.N is expressed in μM of cytochrome c / μM ofoxidase/Sec^{-1}

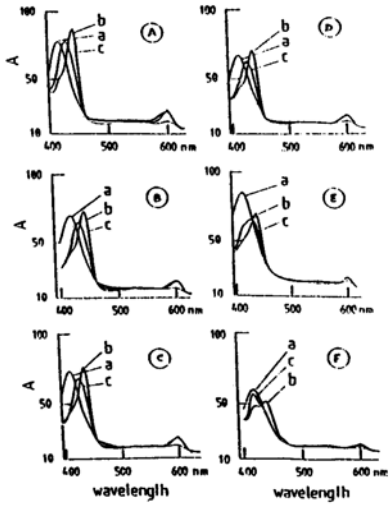

Figure 5: Absolute spectra of chicken cytochrome c oxidase were taken using Parkin Elmer 575 spectrophotometer Cytochrome c oxidase was reduced with sodium dithionite, carbon mono-oxide gas was introduced into fully reduced enzyme and spectrum was superimposed. As shown in Fig 5, (a) oxidised oxidase (b) reduced oxidase (c) reduced oxidase plus carbon-monoxide. Spectra of chicken cytochrome c oxidase shown at different pH values (i.e.) (A) pH 7.4, (B) pH 9.0 (C) pH 9.5 (D) pH 10.0 (E) pH 10.5 (F) pH 11.0.

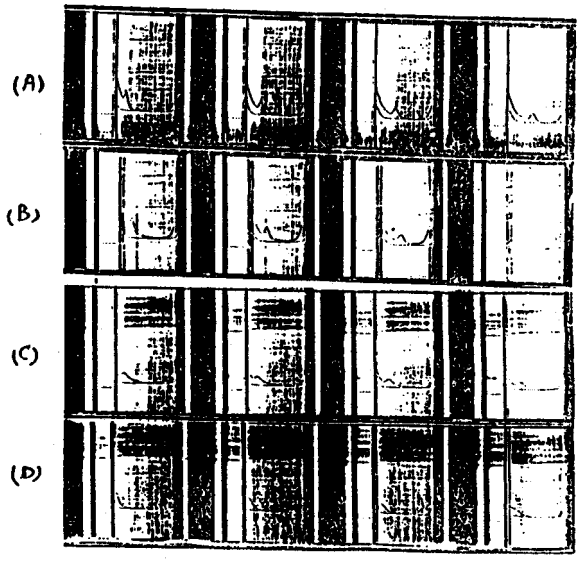

Figure 6: Photographs showing the sedimentation pattern of chicken cytochrome c oxidase in 0.1 M sodium phosphate buffer containing $1^9/6$ Tween 80 at different phi. (A) chicken oxidase in 0.1 M sodium phosphate buffer pH 7.4 containing I% Tween 80 (B) Chicken oxidase in 0.1 M sodium phosphate buffer pH 9.5 containing 1% tween 80 (C) chicken cytochrome c oxidase in 0.1 M sodium phosphate buffer pH 10.0 containing I% Tween 80 (D) Chicken oxidase in 0.1 M sodium phosphate buffer pH 10.8 containing I% Tween 80.

Large peak at meniscus is due to sedimentation of detergent (Tween 80) micelles. Speed of rotor was 52,000 (rpm), temperature 20°C The sedimentation coefficient was calculated from plot Ln vs time (shown in Figure 7).

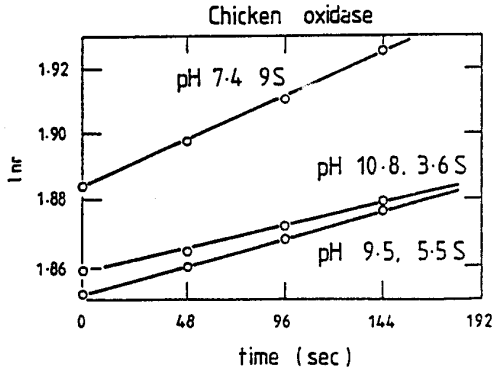

Figure 7: Chicken oxidase (40 to 50 μM) functional units were centrifuged in Beckman Model E ultracentrifuge and three plots are shown in figure at different pH (i.e. 7.4, pH 9.5 and pH 10.8). These were plotted at Lnr (distance from the center of rotor to the sedimentation peak) Versus time. The resulting S values (unconnected for lipids or solvent density) were calculated (see table).

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